

The phosphorylation levels of RyR2 at Ser<sup>2809</sup> and phospholamban at Ser<sup>16</sup> are substantially lower in c-SANC, suggesting that PKA signaling in c-SANC is down-regulated. Additionally, the level of RGS2 protein is substantially lower in c-SANC ( $149.9 \pm 4.0$ ,  $n=100$ ) than in f-SANC ( $201.9 \pm 6.0$ ,  $n=88$ ,  $p<0.001$ ).  $\beta$ -AR stimulation with  $1\mu\text{M}$  isoproterenol for 10min increases phospholamban and RyR2 phosphorylation, accelerates AP and  $\text{Ca}^{2+}$ -transient kinetics, reduces the LCR period, and accelerates the AP firing rate to a similar maximum in c-SANC ( $3.34 \pm 0.05\text{Hz}$ ,  $n=150$ ) and f-SANC ( $3.55 \pm 0.06\text{Hz}$ ,  $n=126$ ). A 2 hour incubation of  $1\mu\text{M}$  isoproterenol enhances RGS2 staining density. A specific PKA inhibitor, PKI, completely inhibits all isoproterenol effects. Overexpression of RGS2 (Ad-RGS2), or overnight pertussis toxin treatment to disable Gi signaling, partially (66% and 85%, respectively) rescues the reduced spontaneous beating rate. These results indicate that a reduction in PKA-dependent  $\text{Ca}^{2+}$ -cycling protein phosphorylation that is Gi-dependent is involved in the reduced spontaneous beating rate of c-SANC, and that this deficit can be reversed by pharmacologic or genetic manipulation.

## 522-Pos

### Spontaneous Beating of Rabbit Sinoatrial Node Cells Requires Basal Protein Kinase C Activity

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Cardiac rhythm is governed by the spontaneous rhythmic excitations of rabbit sinoatrial node cells (SANC), a crucial component of which is cAMP-mediated, PKA-dependent local subsarcolemmal  $\text{Ca}^{2+}$  releases (LCRs) from ryanodine receptors (RyR). LCRs activate inward  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchange current during late diastolic depolarization (DD) leading to the increase in the DD rate and spontaneous SANC beating rate. Here we show that in addition to PKA-dependent phosphorylation, spontaneous SANC beating requires basal PKC activity. In freshly isolated SANC the PKC inhibitor GF109203X,  $10\mu\text{mol/L}$ , suppressed spontaneous SANC beating rate, recorded using perforated patch by  $\sim 2.5$ -fold (from  $154 \pm 7$  to  $60 \pm 14$  beat/min,  $n=6$ ). To study whether IP<sub>3</sub> receptor-mediated  $\text{Ca}^{2+}$  release is involved in the PKC-dependent modulation of the spontaneous SANC beating, IP<sub>3</sub> receptors were inhibited by  $2\mu\text{mol/L}$  2-APB. However, 2-APB produced no significant changes in the spontaneous SANC beating rate suggesting no direct involvement of IP<sub>3</sub>-mediated  $\text{Ca}^{2+}$  release in the spontaneous SANC beating. Confocal imaging of isolated SANC loaded with the  $\text{Ca}^{2+}$  indicator, Fluo-3, demonstrated that PKC inhibition markedly decreased LCR size from ( $8.6 \pm 0.5$  to  $4.0 \pm 0.4\mu\text{m}$ ,  $n=3$ ,  $P<0.01$ ); LCR number per each spontaneous cycle (from  $1.5 \pm 0.3$  to  $0.6 \pm 0.2$ ,  $n=3$ ,  $P<0.05$ ), and prolonged the LCR period, the time from AP-induced  $\text{Ca}^{2+}$  transient to the time of LCR appearance (from  $406 \pm 7$  to  $836 \pm 80$  ms,  $n=3$ ,  $P<0.05$ ). The increase in the LCR period accompanied the increase in the spontaneous SANC cycle length, strongly suggesting that LCRs could be the major target of the basal PKC activity. Thus, our data show for the first time that the basal PKC-dependent activity regulates spontaneous SANC beating rate through modulation of LCRs from RyR, while IP<sub>3</sub> receptors make no substantial contribution in this effect.

## 523-Pos

### Mechanisms Underlying Spontaneous Beating in Human Embryonic Stem Cell-Derived Cardiomyocytes

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Cardiac myocytes derived from human embryonic stem cells (hESC-CMs) are increasingly considered a promising therapy for a variety of pathologies, but many questions persist regarding the physiology of these cells and how it changes during differentiation. We sought to determine the mechanisms underlying spontaneous beating in hESC-CMs derived from HES2 line. Enrichment for developing cardiomyocytes was achieved by exposing differentiating Embryoid Bodies (EBs) to the following growth factors: BMP4, DKK and VEGF, and DKK, VEGF and bFGF. By day 20, >85% of EBs were spontaneously beating and roughly 70% of all cells were expressing troponin T. To monitor intracellular calcium ( $\text{Ca}^{2+}$ ), EBs from various time points were plated on cover slips and imaged with a laser scanning confocal microscope. Recordings of  $\text{Ca}^{2+}$  transients in hESC-CMs at different stages of differentiation revealed the following features: 1) rate of spontaneous beating increased, and  $\text{Ca}^{2+}$  transients decayed more quickly, in older (> 45 day) compared with younger (< 45 day) hESC-CMs; 2) coordinated spontaneous beating was abolished in 100% of EBs when external  $\text{Na}^{+}$  was replaced with  $\text{Li}^{+}$ ; 3) reducing extracellular [ $\text{Ca}^{2+}$ ] to 0.5 mM caused slower spontaneous beating; 4) spontaneous beating was abolished in most (>75%) EBs when 20 mM caffeine depleted sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ ; 5) field-stimulation induced  $\text{Ca}^{2+}$  transients recorded

in 20 mM caffeine decayed more slowly than control  $\text{Ca}^{2+}$  transients, and the difference in decay rate was more pronounced in older hESC-CMs. Together these results are consistent with a model in which spontaneous SR  $\text{Ca}^{2+}$  release induces inward  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchange current, and the membrane depolarization provided by this current initiates action potentials. Differentiation time increases the importance of SR  $\text{Ca}^{2+}$  cycling in the function of these cells, suggesting a more mature phenotype in older compared with younger hESC-CMs.

## 524-Pos

### Competitive NFAT Bottleneck For Transcriptional Activation of Endogenous $\text{Ca}^{2+}$ ATPASE (SERCA2) in Adrenergic Hypertrophy

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A prominent feature of cardiac hypertrophy and failure is reduced SERCA2 expression and deficient  $\text{Ca}^{2+}$  signaling. To uncover a causal relationship between hypertrophy and downregulation of SERCA2, we use neonatal rat cardiac myocytes where SERCA2 transcription can be increased by exposure to 10 nM thapsigargin (TG), which allows cytosolic  $\text{Ca}^{2+}$  rise and calmodulin activation of calcineurin (CN). This SERCA2 rise is markedly reduced by cyclosporine (CsA), which inhibits CN phosphatase, and increased by KN-93, a calmodulin activated kinase (CAMKII) inhibitor which relieves CAMKII dependent phosphorylation and inhibition of CN. These CsA and KN-93 effects are also produced on TG enhanced luciferase expression, under the control of an NFAT (Nuclear factor of activated T Cells) dependent promoter. We conclude that NFAT dephosphorylation is a limiting factor for SERCA2 transcription. Exposure of myocytes to PE yields adrenergic hypertrophy, with rise of Atrial Natriuretic Factor (ANF) transcript, protein incorporation of <sup>14</sup>C-phenylalanine and fluorescent staining of actin, while SERCA2 is downregulated. The adrenergic response can be reproduced by direct stimulation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate, indicating that inhibition of glycogen synthase kinase (GSK3 $\beta$ ) by PKC and consequent reduced NFAT phosphorylation, as well as histone deacetylase (HDAC) phosphorylation by PKC activated MAPK, are involved in the mechanism of adrenergic hypertrophy. The hypertrophy response is markedly reduced by CN inhibition by CsA, indicating that CN dephosphorylation and nuclear import of NFAT play an important role in the development of hypertrophy, and SERCA2 downregulation is produced by competitive utilization of NFAT by the hypertrophy program. Interestingly, CAMKII inhibition limits the development of hypertrophy, emphasizing a rate limiting role of CAMKII dependent phosphorylation and nuclear export of HDAC in the extensive transcriptional hypertrophic program (Supported by 5 R01 HL069830-08).

## 525-Pos

### Differential Regulation of $\beta$ -Adrenergic Signaling Via Phosphoinositide-3-Kinase- $\gamma$

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We have recently shown that phosphoinositide-3-kinase- $\gamma$  (PI3K $\gamma$ ) modulates cardiomyocyte contractility by locally regulating sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  load via the type 4 phosphodiesterase (PDE4) (Kerfant Circ. Res. 2007). In addition, we have also shown that SR  $\text{Ca}^{2+}$  load and contractile are enhanced in PI3K $\gamma$  knockout (KO) mice both at baseline in the context of non-selective  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation (Kerfant Circ. Res. 2004). Since PI3K $\gamma$  is activated by the  $\beta\gamma$  subunit of G-proteins which has also been shown to affect  $\beta$ -AR signaling in cardiomyocytes, we asked whether PI3K $\gamma$  differently affects  $\beta_1$ -AR versus  $\beta_2$ -AR signaling and whether PDE4 underlies these differences. We used L-type  $\text{Ca}^{2+}$  currents ( $I_{\text{CaL}}$ ) and  $\text{Ca}^{2+}$  transients to assess the effects of  $\beta$ -AR stimulation. In isolated murine cardiomyocytes, specific  $\beta_1$ -AR stimulation increased  $I_{\text{CaL}}$ , and  $\text{Ca}^{2+}$  transient amplitudes in both wild-type (WT) and KO cells, although the peak transient response was more pronounced in the latter. Concomitant inhibition of PDE4 with  $10\mu\text{M}$  rolipram had no effect on  $\text{Ca}^{2+}$  transient amplitudes in KO cells while in WT cells, transient amplitudes were further increased to levels observed in KO's. While specific  $\beta_2$ -AR stimulation increased  $\text{Ca}^{2+}$  transient amplitudes in both WT and KO cells (not to the same extent as  $\beta_1$ ), this increase was not accompanied by any changes in  $I_{\text{CaL}}$ . Furthermore, while transients were larger in KO cells, rolipram reduced the parity between these groups. In summary, specific activation of the  $\beta_1$  pathway enhances cardiac  $\text{Ca}^{2+}$  handling in a PI3K $\gamma$ /PDE4-dependent manner. Conversely,  $\beta_2$ -AR activation has little impact on  $I_{\text{CaL}}$ , plays a smaller role in  $\text{Ca}^{2+}$  handling, but also appears to be partially regulated by PDE4. Taken together, these results suggest that PI3K $\gamma$  plays an important role in both  $\beta_1$ , and  $\beta_2$ -AR signaling.